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Cell surface glycosaminoglycans are not involved in the adherence of *Helicobacter pylori* to cultured Hs 198.St human gastric cells, Hs 746T human gastric adenocarcinoma cells, or HeLa cells

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Hs 198.St cells (a line derived from normal human gastric tissue), Hs 746T cells (a line derived from human gastric adenocarcinoma), and HeLa cells were used together with ^{3}H-labelled *Helicobacter pylori*, strain NCTC 11637 to determine if cell surface glycosaminoglycans could act as initial receptors for adherence of the bacteria. Although as much as 40% of the ^{3}H-labelled bacteria adhered to monolayers of the cultured cells, removal of glycosaminoglycans by prior treatment of the cells with heparitinase, heparinase, or chondroitin ABC lyase had no effect in modifying the adherence. Prior addition of heparan sulfate, heparin, or chondroitin/dermatan sulfate to bacteria had no effect on adherence, nor were bacteria released when these same glycosaminoglycans or these same enzymes were added to cultures already containing adherent bacteria. These results indicated that neither heparan sulfate nor chondroitin/dermatan sulfate are involved as receptors in the initial adherence step of *H. pylori* to these cultured cells.

Keywords: *Helicobacter pylori*, heparan sulfate, chondroitin sulfate, proteoglycan, glycosaminoglycan, gastric cells

Introduction

Helicobacter pylori is a gastric pathogen which is associated with chronic gastritis and gastric ulcer disease [1]. In addition, there appears to be a strong association between *H. pylori* infection and the development of a variety of gastric carcinomas [2–5]. These bacteria have been found *in vivo* to be adherent to gastric epithelial cells, but are not found in the duodenum or the esophagus except in areas of gastric metaplasia [6]. Adherence of the bacteria to gastric epithelial cells would appear to be the initial step in bacterial infection [7], suggesting that there may be specific receptors on the gastric cell surface. Several candidate receptors for adherence have been proposed, including N-acetylneuraminyllactose [8], phosphatidylethanolamine [9], GM3 ganglioside [10], sulfatide [10–12], Lewis^{b} antigen [13], and glycosaminoglycans such as heparan sulfate and chondroitin sulfate [14–16].

Proteoheparan sulfate and chondroitin sulfate are found on most if not all mammalian cell surfaces [17]. Proteoheparan sulfate is thought to play a role in the initial adherence of several infectious microbes [18–21], and proteochondroitin sulfate has been shown to be a receptor for *plasmodium falciparum*-infected erythrocytes [22]. The binding of small amounts of heparan sulfate glycosaminoglycan to cultures of *H. pylori* has been described [14, 15], and more recently, it has been reported that cell surface heparan sulfate on murine macrophages mediated an adherence of *H. pylori* [16]. However, there have been no reports concerning whether or not heparan sulfate or chondroitin sulfate serve as receptors for *H. pylori* adherence to living gastric epithelial cells.

In order to examine this possibility, we now have used *in vitro* models of *H. pylori* adherence to cultured human

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cells, utilizing Hs 198 *St* cells (derived from normal gastric tissue) and Hs 746T cells (derived from gastric adenocarcinoma). In addition we have used HeLa cells (derived from cervical adenocarcinoma) as a non-gastric control cell. Although adherence of bacteria took place with all three types of cells, glycosaminoglycans did not appear to be involved as receptors.

Materials and methods

Sodium [^{35}S]sulfate (carrier free) and [^{3}H]proline (19.9 Ci mmol^{-1}) were purchased from New England Nuclear (Boston, MA). Heparin and trypsin were purchased from Sigma Chemical Co. (St. Louis, MO). Heparan sulfate, chondroitin sulfate, dermatan sulfate, chondroitin ABC lyase, heparinase, and heparitinase were purchased from Seikagaku America, Inc. (Rockville, MD). Crude enzyme was prepared from *Flavobacterium heparinum* (a gift from Dr A. Linker, Salt Lake City, UT) as previously described [23, 24]. All other chemicals were of analytical grade and were purchased from various commercial sources.

Helicobacter pylori growth conditions

Helicobacter pylori strain NCTC 11637 was obtained from the American Type Culture Collection (ATCC, Rockville, MD). This is the same strain as the Culture Collection, University of Gothenburg (CCUG), strain 17874 [25], which has been used most frequently to examine attachment of heparan sulfate [15, 16]. Bacteria were grown as previously described [26] on trypticase soy agar plates (BBL Microbiology systems, Cockeysville, MD) containing 5% defibrinated sheep blood at 37 °C for 5 days in a GasPak jar with Catalyst Replacement Charges (BBL) and CampyPaks (BBL). Scrapings were transferred to 10 ml of brucella broth (BBL) containing trimethoprim (5 mg l^{-1}) and vancomycin (10 mg l^{-1}) supplemented with 10% foetal bovine serum (Hyclone Laboratories Inc., Logan, UT), and incubated at 37 °C in a BIO-BAG TYPE Cfj (BBL) with Microaerophilic Generator (BBL) which is designed to provide 5–15% O_2, 5–12% CO_2. Bacteria were grown for 48 h with shaking at 150 rpm, and characterized as *H. pylori* on the basis of morphology under phase-contrast microscopy, Gram staining, and their production of urease, oxidase, and catalase [27, 28]. Aliquots in bovine serum-containing media were stored at −70 °C prior to use as described previously [28]. When bacteria were incubated in air at 37 °C, there was no growth of any bacteria, confirming the lack of contamination with other organisms.

Quantitation of bacteria in suspension was determined visually by light microscopy of aliquots, and by optical density measurements at 600 nm. Appropriate dilutions of the bacterial suspensions were spread on Belo Horizonte medium [29], on which *H. pylori* produce red colonies. Colony-forming units (CFU) were counted after incubation of plates for 5 days at 37 °C under microaerophilic conditions.

Radiolabelling of H. pylori

[^{3}H]Proline (5 μCi ml^{-1}) was added to growth medium at the time of bacterial inoculation [30, 31]. Cultures were incubated as above for 48 h and then harvested by centrifugation at 3000 × g for 15 min, washed three times in 0.01 M phosphate-buffered saline (PBS), pH 7, and suspended in PBS to a final concentration of 10^9 CFU per ml. Aliquots were assayed for radioactivity with a β-scintillation spectrometer.

Cell culture

Hs 198.St normal human stomach cells (ATCC CRL 7155), Hs 746T human stomach carcinoma cells (ATCC HTB 135), and HeLa human cervix carcinoma cells (ATCC CCL 2) were obtained from ATCC. All cells were cultured at 37 °C with 5% CO_2 in 98% relative humidity. Hs 198.St Cells were routinely grown in DMEM (GIBCO Laboratories Inc., Grand Island, NY) supplemented with 10% (vol/vol) foetal bovine serum (Hyclone). Hs 746T Cells were routinely grown in DMEM with 4.5 g l^{-1} glucose supplemented with 10% (vol/vol) foetal bovine serum. HeLa cells were grown in MEM plus nonessential amino acids (Sigma) and supplemented with 10% (vol/vol) foetal bovine serum. Penicillin G (100 U ml^{-1}) and streptomycin (0.1 mg ml^{-1}) were added to all cell cultures. As with other established cell lines from similar sources, there was no indication of any mucin production by either the HS 198.St or the Hs 746T cells.

Adherence assay

These were performed as previously described [31]. In typical experiments, 2 × 10^5 cells in 1 ml of medium were seeded into separate wells of a 24-well culture cluster (COSTAR Cambridge, MA) and incubated for 24 h at 37 °C to obtain semi-confluent monolayers. After washing three times with PBS, 0.9 ml of PBS, pH 7, supplemented with 0.2% bovine serum albumin was added. ^{3}H-Labelled bacteria (0.1 ml usually containing 5 × 10^7 CFU) were then added and the clusters incubated for 90 min at 37 °C, 20 °C, or 4 °C at pH 5 to pH 8 under the microaerophilic conditions used for growth of the bacteria. The cell monolayers were washed five times in PBS with constant agitation and then incubated in 0.1% (wt/vol) SDS-0.2 M NaOH to remove and suspend the cells. The washes (non-adherent bacteria) and the suspended cells (cell-adherent bacteria) were subjected to scintillation counting and the proportions of adherent organisms were calculated.

Non-specific binding of *H. pylori* to polystyrene was examined by incubation of an equal number of radiolabelled bacteria in wells that did not contain cultured cells. Non-specific binding (usually less than

0.2% of the added bacteria) was subtracted from all experimental values. Cell viability in comparable wells was determined by Trypan Blue exclusion and by direct phase contrast microscopy at the end of each experiment.

Pretreatment of cell monolayers with glycosaminoglycan lyases

Glycosaminoglycan lyases (chondroitin ABC lyase, heparinase, heparitinase, or crude *F. heparinum* lyase) 1 U in 1 ml of PBS, pH 7, containing 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$ were added to the cell layers, and incubated for 60 min at 37 °C as described previously [23, 24]. After removing the lyases and washing twice with PBS (4 °C), ^{3}H-labelled *H. pylori* was added and incubated with the monolayers for 90 min at 37 °C under microaerophilic conditions. The proportions of adherent counts were determined as described above. None of the lyases caused any detachment of cultured cells from the monolayers.

Pretreatment of bacteria with glycosaminoglycans and trypsin

^{3}H-labelled *H. pylori*, 5×10^7 CFU, were incubated at 20 °C for 1 h with 0.1 mg of unlabelled chondroitin sulfate, dermatan sulfate, heparin, or heparan sulfate in a total volume of 2 ml. The treated cells were washed with PBS, suspended in 0.1 ml of PBS, and used in the adherence assay described above. Similar suspensions of ^{3}H-labelled bacteria were also incubated at 37 °C for 1 h with 0.1 mg of trypsin in 2 ml of 0.05 M Tris-HCl buffer, pH 7, containing 5 mM $CaCl_2$, as described previously [14]. Cells were washed twice with PBS, suspended in PBS and used in the adherence assay described above.

Labelling of cell surface glycosaminoglycans

Cell cultures were incubated for 48 h as above in medium supplemented with [^{35}S]sulfate (50 μCi ml^{-1}) with a sulfate concentration of 0.8 mM. Cells were rinsed repeatedly with PBS (4 °C) containing $CaCl_2$ and $MgCl_2$ to remove free [^{35}S]sulfate.

Results

Adherence of ^{3}H-labelled H. pylori to cultured cells

After examining a number of gastric cell lines we selected Hs 198.St and Hs 746T cells for ease of culture and for their tight attachment to culture dishes. We utilized the extensively-studied non gastric HeLa cell line as a reference, since adherence of *H. pylori* to these cells has been reported [32]. We also attempted the use of KATO III gastric epithelial cells since they have been used to study adherence of *H. pylori* [31, 33, 34], although not to glycosaminoglycans on these cells. We found use of the KATO III cells to be difficult since they fail to attach to culture dishes [35].

The percentage of ^{3}H-labelled *H. pylori* adhering to Hs 198.St, Hs 746T, and HeLa cells (Fig. 1) remained nearly constant at approximately 13%, 38%, and 25% respectively when 5×10^5 to 5×10^7 CFU of bacteria were applied, suggesting that there ws a large subpopulation of bacteria incapable of adherence. This was confirmed when essentially none of the non-adherent bacteria were capable of adhering to Hs 746T cells when reapplied to these cells. However, when 5×10^8 CFU of bacteria were added to each of the three cell lines (Fig. 1), the percentage of adherent bacteria decreased, indicating that the adherence was saturable, and suggesting that specific receptors were involved. In all further experiments, 5×10^7 CFU of bacteria were used. After Giemsa staining of washed subconfluent monolayers, the bacteria were seen to be directly in contact with the cell surfaces.

A time course for adherence of *H. pylori* to the cell monolayers is shown in Fig. 2. Rapid adherence to all three cell lines occurred within 30–60 min of incubation, and then continued at a slower rate for the next 3 h. There was no significant increase in adherence after an additional 20 h. Essentially all cells continued to exclude trypan blue, and cells could be subcultured easily even after a total 24 h under the microaerophilic conditions. Change of pH from 5 to 7 did not affect adherence to any great degree, but the adherence was markedly diminished when incubations were at 20 °C and 4 °C rather than 37 °C (Table 1), suggesting that some modifications of cell surface receptors or changes in metabolism were affecting the adherence.

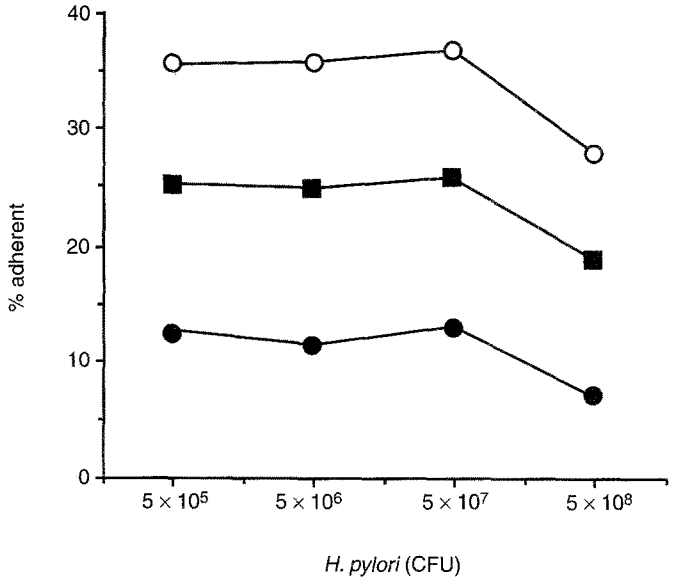

Figure 1. Relationship of adherence to the number of bacteria applied. Subconfluent cell monolayers of Hs 198.St (●), Hs 746T (○), and HeLa (■), were incubated with varying amounts of ^{3}H-labelled *H. pylori* at 37 °C. Bacterial adherence was determined as described in Materials and Methods. Each data point represents the means of for three replicates with an SD for each <9%.

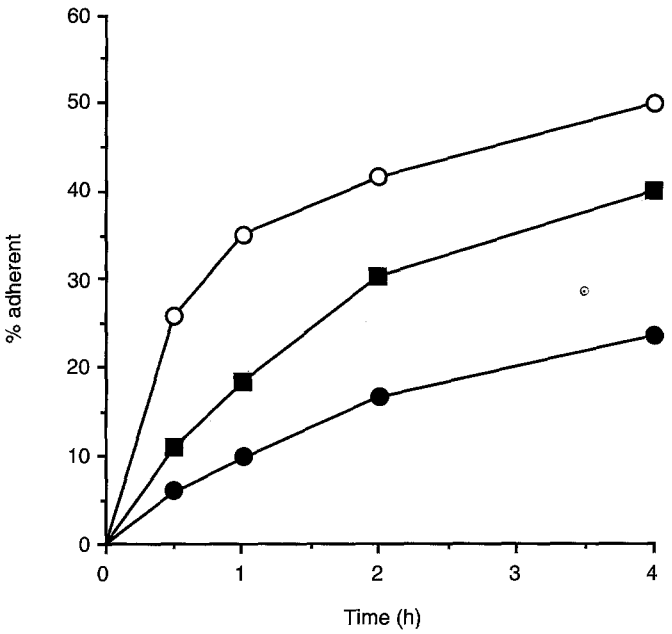

Figure 2. Effect of incubation time on bacterial adherence. Subconfluent cell monolayers of Hs 198.St (●), Hs 746T (○), and HeLa (■), were incubated with ^{3}H-labelled *H. pylori* at 37 °C. Bacterial adherence was determined as described in Materials and Methods. Each data point represents the means of for three replicates with an SD for each <7%.

Table 1. Effect of temperature on adherence to cell monolayers

	*Per cent of added bacteria adherent**		
	Hs198.St	*Hs 746T*	*HeLa*
4 °C	2.4	2.6	6.5
20 °C	5.5	22.5	18.4
37 °C	15.6	35.2	27.8

*Mean of triplicate measurements, SD for each <6%.

Adherence to cultured cells pretreated with glycosaminoglycan lyases

Removal of cell surface glycosaminoglycans by pretreatment of monolyaers with chondroitin ABC lyase (capable of degrading chondroitin/dermatan sulfate), heparinase (capable of degrading heparin), heparitinase (capable of degrading heparan sulfate), and *F. heparinum* enzyme (capable of degrading all these glycosaminoglycans) had no effect on adherence of ^{3}H-labelled *H. pylori* (Table 2). Furthermore no bacteria were released when these same enzymes were added to cell monolayers that already contained adherent bacteria (data not shown). These results are in contrast to a previous report that heparitinase pretreatment of mouse macrophage monolayers caused a 33% decrease in the adherence of *H. pylori* [16].

We then used the same enzymes on cell monolayers that had been preincubated with [^{35}S]sulfate in order to form [^{35}S]proteoglycans. Results shown in Table 3 demonstrate that these enzymes were fully effective in removing cell surface heparan [^{35}S]sulfate, chondroitin/dermatan [^{35}S]sulfate, or both.

Table 2. Adherence to cell monolayers pretreated with glycosaminoglycan lyases

	*Per cent of added bacteria adherent**		
Enzyme	*Hs198.St*	*Hs 746T*	*HeLa*
None	9.9	39.6	24.6
Chondroitin ABC lyase	9.9	39.8	22.3
Heparinase	10.2	36.8	22.3
Heparitinase	9.9	37.6	23.6
Crude *F. heparinum*	10.1	39.0	24.8

*Mean of triplicate measurements, SD for each <6%.

Table 3. Enzymatic treatment of ^{35}S-labelled cell monolayers

	*^{35}S-labelled material released cpm per culture well**		
Enzyme	*Hs 198.St*	*Hs 746T*	*HeLa*
None	6000	12 000	4400
Chondroitin ABC lyase	19 000	15 000	15 300
Heparinase	8000	21 500	14 800
Heparitinase	13 000	19 000	15 300
Crude *F. heparinum*	22 400	30 000	18 300

*Mean of triplicate measurements, SD for each <12%.

Adherence of H. pylori pretreated with glycosaminoglycans or trypsin

To substantiate the previous results, adherence assays were undertaken after pretreatment of ^{3}H-labelled *H. pylori* with chondroitin/dermatan sulfate, heparin, or heparan sulfate (Table 4). There was no effect on adherence nor any release of bacteria when these same glycosaminoglycans were added to monolayers already containing adherent bacteria (data not shown). These results are in contrast to the reported 67% decrease in adherence of *H. pylori* to mouse macrophages pretreated with heparin or

Table 4. Adherence to cell monolayers after pretreatment of bacteria with glycosaminoglycans or trypsin

	*Per cent of added bacteria adherent**		
	Hs198.St	*Hs 746T*	*HeLa*
No treatment	8.8	35.3	18.3
Chondroitin sulfate	12.8	35.7	17.9
Dermatan sulfate	9.1	37.1	21.6
Heparin	8.0	35.0	21.0
Heparan sulfate	9.6	37.3	21.3
Trypsin	6.0	15.0	4.4

*Mean of triplicate measurements, SD for each <3%.

heparan sulfate [16]. Pretreatment of bacteria with trypsin, reduced the adherence of bacteria markedly (Table 4).

Thus despite the suggestions in the literature that heparan sulfate may be a factor in the initial adherence of *H. pylori* to gastric cells, we have found no evidence that such is the case. It now seems clear from recent reports [12] that the initial attachment to sulfated substances is to sulfatides and not to proteoglycans of the cell surface. It should be noted however, that our experiments concerning the role of glycosaminoglycans in adherence were for 90 min, while *H. pylori* has the ability to adhere to stomach for the prolonged periods necessary to cause peptic ulceration and to increase the risk of gastric cancer [3]. In addition, we have not ruled out the possibility that heparan sulfate or chondroitin/dermatan sulfate might act as receptors in later steps as in the binding of pathogens such as *Trypanosomes* [19] or malaria parasites [22], where the primary attachments are to other acceptors, with heparan sulfate or chondroitin sulfate being involved only at a secondary stage.

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